Am. J. Hum. Genet. 56:368–373, 1995

Achondroplasia Is Defined by Recurrent G380R Mutations of FGFR3

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Summary

Genomic DNA from 154 unrelated individuals with achondroplasia was evaluated for mutations in the fibroblast growth factor receptor 3 (FGFR3) transmembrane domain. All but one, an atypical case, were found to have a glycine-to-arginine substitution at codon 380. Of these, 150 had a G-to-A transition at nt 1138, and 3 had a G-to-C transversion at this same position. On the basis of estimates of the prevalence of achondroplasia, the mutation rate at the FGFR3 1138 guanosine nucleotide is two to three orders of magnitude higher than that previously reported for tranversions and transitions in CpG dinucleotides. To date, this represents the most mutable single nucleotide reported in the human genome. The homogeneity of mutations in achondroplasia is unprecedented for an autosomal dominant disorder and may explain the relative lack of heterogeneity in the achondroplasia phenotype.

Introduction

Achondroplasia (MIM 100800) is the most common form of short-limbed dwarfism in humans and is transmitted as an autosomal dominant trait with complete penetrance (Jones 1988; Gorlin et al. 1990). The prevalence of achondroplasia is estimated to be between 1/15,000 and 1/77,000 (Murdoch et al. 1970; Gardner 1977; Oberklaid et al. 1979; Andersen and Hauge 1989; Stoll et al. 1989). Sporadic cases, i.e., new mutations, are thought to account for 80%–90% of all cases and have been associated with increased paternal age (Murdoch et al. 1970; Oberklaid et al. 1979; Stoll et al. 1989).

Received September 20, 1994; accepted for publication November 7, 1994.

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0002-9297/95/5602-0002$02.00

The clinical features of achondroplasia are evident at birth, and the diagnosis is usually unambiguous. The classic physical features include proximal shortening of the extremities, genu varum, trident hand, limitation of elbow extension, exaggerated lumbar lordosis, megalencephaly, and characteristic facies with frontal bossing and midface hypoplasia (Jones 1988; Gorlin et al. 1990) The radiological features include small cuboid vertebral bodies with progressive narrowing of the caudal interpedicular distance, lumbar lordosis, thoracolumbar kyphosis with occasional anterior beaking of the first and second lumbar vertebrae, small iliac wings with a narrow greater sciatic notch, and short tubular bones with metaphyseal flare and cupping (Langer et al. 1967).

Medical complications associated with heterozygous achondroplasia include delayed motor milestones, communicating hydrocephalus, cervicomedullary compression secondary to a small foramen magnum, apnea, frequent sinusitis and otitis media, spinal stenosis, and numerous orthopedic problems (Wynne-Davies et al. 1981; Nelson et al. 1988; Gorlin et al. 1990). Mortality is particulary increased for people with achondroplasia from birth to 4 years and in the late 4th to 5th decades of life (Hecht et al. 1987). Overall intelligence is normal, and the degree of severity and age at onset of associated medical problems is highly variable among individuals. Many have little or no morbidity directly attributable to achondroplasia.

When both parents have achondroplasia, there is a 25% risk that an offspring will be homozygous. Children with two achondroplasia alleles have a severe phenotype with characteristic radiological features, a small thoracic cage with respiratory insufficiency, brainstem compression, and severe neurologic deficits. Most usually die within the 1st year of life (Pauli et al. 1983; Hecht et al. 1986).

Recent genetic studies have localized the gene for achondroplasia to human chromosome 4p16.3 (Francomano et al. 1994; LeMerrer et al. 1994; Velinov et al. 1994) and limited the candidate region to a 2.5-Mb segment extending from D4S43 to the telomere (Francomano et al. 1994). More recently, Rousseau et al. (1994) and Shiang et al. (1994) have identified mutations specific to achondroplasia in a fibroblast growth factor receptor gene, FGFR3, which lies within the achondroplasia candidate region. Altogether

they analyzed 39 separate achondroplasia chromosomes and found 37 G-to-A transitions and two G-to-C transversions at nt 1138 of the FGFR3 cDNA sequence. Both of the mutations result in a glycine-to-arginine substitution at codon 380 (G380R) in the transmembrane domain of FGFR3.

The fibroblast growth factor receptors (FGFRs) are a family of four transmembrane glycoproteins (~115–150 kD) that bind fibroblast growth factors (FGFs) with low capacity and high affinity. They are highly homologous, and all contain three extracellular immunoglobulin-like FGF-binding domains, a transmembrane domain, and an intracellular tyrosine kinase domain. FGFR1, FGFR2, and FGFR3 undergo alternative exon splicing in the proximal immunoglobulin-like domain to generate variant receptor forms with different affinities for specific FGFs (Johnson and Williams 1993; Partanen et al. 1993; Chellaiah et al. 1994). The FGFRs have unique patterns of expression during embryogenesis, suggesting that they mediate different functions of FGFs during development. In mouse organogenesis, fgfr-3 is expressed in the germinal epithelium of the neural tube, in a diffuse, glial pattern in adult brain and in differentiating hair cells of the cochlear duct. Outside the nervous system, fgfr-3 is expressed at high levels in cartilage rudiments of developing bone and, exclusive of fgfr-1 and fgfr-2, in resting cartilage during endochondral ossification (Peters et al. 1993).

Using methods developed by Shiang et al. (1994) to screen for the occurrence of either of the G380R FGFR3 mutations in genomic DNA, we analyzed our collection of DNA from patients with achondroplasia. An 1138 G-to-A transition or 1138 G-to-C transversion was found in all individuals with typical achondroplasia and was not detected in 1 atypical case or 147 normal controls.

Subjects, Material, and Methods

Patients with achondroplasia were identified through the Medical Genetics Clinics at the Johns Hopkins Hospital, the University of Texas Medical School at Houston, the Helsinki University Hospital, and the membership of the Little People of America. They include multigenerational families described elsewhere (Francomano et al. 1994) and sporadic cases from diverse racial and ethnic backgrounds (including Hispanic, African, northern European, Mediterranean, and Finnish).

Genomic DNA was prepared from whole blood or lymphoblast cell lines, either as described elsewhere (Kunkel et al. 1977) or by protein precipitation as follows: Whole blood collected in standard EDTA tubes was mixed with 3 vol of a solution consisting of 150 mM NH_4Cl, 2 mM Na_2EDTA, 7 mM $NaHCO_3$ (pH 6.5) and allowed to sit at room temperature for 5 min with occasional gentle mixing. Leukocytes were separated from the lysed red blood cells (RBCs) by centrifugation (2,000 *g*; 10 min). The leukocyte pellet was resuspended by vortexing in the small amount of residual buffer remaining after decanting the supernatant and then dissolved in a volume equal to the original blood volume of a lysis solution consisting of 10 mM Tris HCl (pH 7.4), 20 mM Na_2EDTA, and 1% SDS. When the pellet was fully dissolved, 0.05 mg of DNAse-free RNAse/ml was added, and incubation was continued for 15 min at 37°C. Proteins were precipitated by adding 6 M ammonium acetate to a final concentration of 1.5 M, followed by vigorous vortexing for 10 s and centrifugation (2,000 *g*; 10 min). DNA was precipitated with isopropanol, was washed with 70% ethanol, and was resuspended in 10 mM Tris HCl, 1 mM EDTA (pH 8.0) buffer. Isolation of DNA from lymphoblast cells was identical to that of whole blood, except the RBC lysis step was omitted, and lymphoblasts ($\sim 2.0 \times 10^7$) were suspended in 5 ml of the SDS lysis buffer.

Reaction conditions and FGFR3 PCR primers used for amplification of genomic DNA were described by Shiang et al. (1994). PCR products were digested with restriction enzymes according to manufacturer's instructions and were analyzed on 2% NuSieve/1% agarose slab gels in standard 89 mM Tris, 89 mM boric acid, 2 mM EDTA (pH 8.0) buffer. DNA sequencing of PCR products was performed using the BRL cycle sequencing kit and the same primers used for PCR amplification of genomic DNA.

Results

The 1138 G-to-A transition and G-to-C transversion create novel restriction sites (*Sfc*I and *Msp*I) in the FGFR3 transmembrane-domain nucleotide sequence. These mutations can be detected by PCR amplification of a 164-nt portion of FGFR3 that includes the transmembrane sequence, followed by digestion with either enzyme (Shiang et al. 1994; see fig. 1). Using this method, we screened genomic DNA from a collection of 193 individuals with achondroplasia and 147 unaffected family members. The 193 individuals with achondroplasia represent 114 sporadic cases and 79 inherited cases (including 4 homozygotes) and account for 154 distinct achondroplasia chromosomes. Of the 154 unrelated achondroplasia chromosomes represented in this patient group, 150 had the FGFR3 1138 G-to-A mutation, and 3, all sporadic cases, had the 1138 G-to-C transversion (see table 1). In 42 sporadic cases the de novo occurrence of either mutation was verified by analysis of DNA from both parents. Cosegregation of achondroplasia with the 1138 G-to-A mutation was observed in 39 informative meioses from 25 families. One patient, a severely affected, atypical, sporadic case, did not have either mutation. Sequencing of the FGFR3 transmembrane domain in this individual revealed normal sequence of both alleles (data not shown). None of the 147 unaffected family members demonstrated either the 1138 G-to-A or G-to-C mutation. Our data, added to those of Rous-

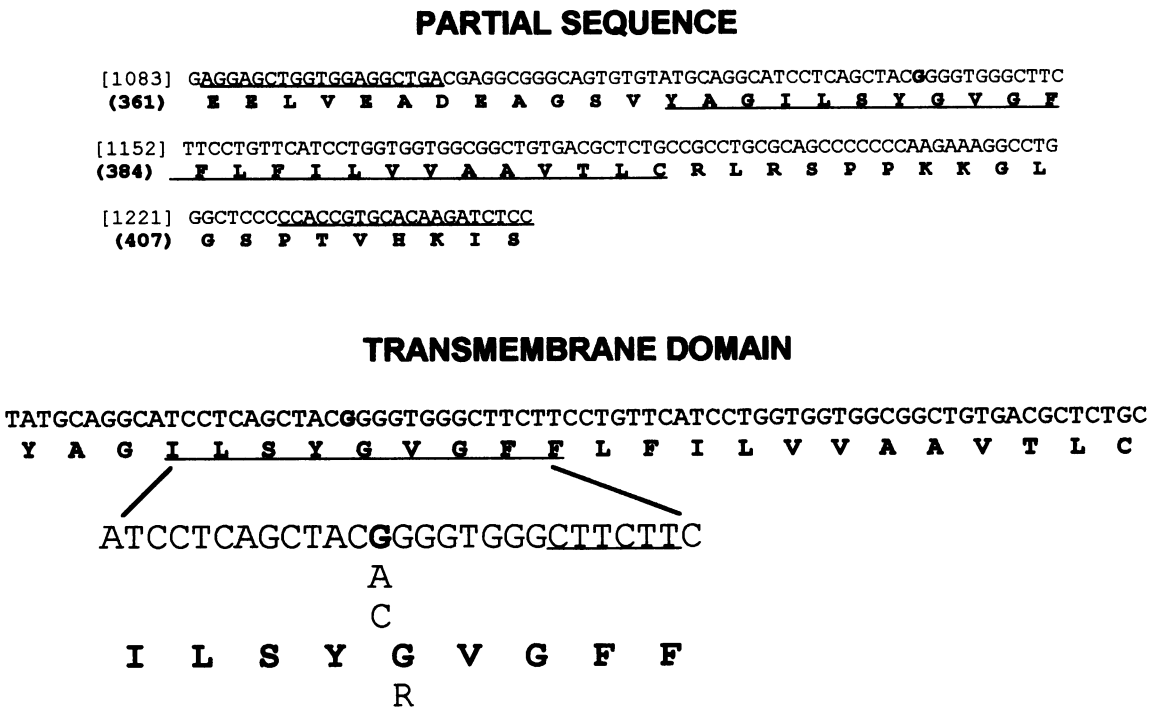

Figure 1 Partial sequence of the FGFR3 cDNA, depicted with the amino acid translation according to Thompson et al. (1991) and Keegan et al. (1991). Locations of the PCR primers used in this study are underlined in the DNA sequence, and the transmembrane domain is underlined in the amino acid sequence. The sequence of the transmembrane domain is shown with the area of enlargement underlined. Nucleotide 1138 is depicted in boldface type, and the two observed mutations are drawn directly underneath, as is the amino acid substitution. The two topoisomerase-I recognition-site trinucleotides (CTT) are underlined.

seau et al. (1994) and Shiang et al. (1994), suggest that >99% of achondroplasia is caused by an FGFR3 G380R mutation.

Discussion

The results of this study confirm and extend the findings of Rousseau et al. (1994) and Shianget et al. (1994) that the FGFR3 G380R mutation is responsible for the achondroplasia phenotype in most, if not all, cases. We have demonstrated that the 1138 G-to-A transition represents 97% of achondroplasia mutations and that the 1138 G-to-C transversion accounts for >2%. The one patient who did not demonstrate either mutation is an unusual, sporadic case who presented with a severe phenotype resembling homozygous achondroplasia. She also had several radiographic features not normally seen in achondroplasia, including posterior rotation of the hips and exaggerated bowing of the lower extremities. Nevertheless, her physical and radiographic features were more consistent with achondroplasia than any other known skeletal dysplasia.

It is possible that the FGFR3 G380R amino acid substitution is the only mutation that causes the achondroplasia phenotype and that other mutations in FGFR3 either are silent, result in other phenotypes, or are not compatible with survival. Given the consistency of the achondroplasia phenotype, in terms of physical appearance and radiographic findings, perhaps it should not be surprising that a single mutation is responsible. However, the variation in the severity of medical complications among individuals with achondroplasia cannot be attributed to allelic heterogeneity, since nearly every individual has the same allele. Given the high degree of homogeneity of the achondroplasia mutation, prenatal diagnosis for the homozygous condition should be extremely reliable and readily available to all couples at risk.

Recently, mutations in two other FGFRs have been found in three distinct inherited disorders of skeletogenesis. FGFR2 mutations have been found in patients with Crou-

Table 1

FGFR3 Transmembrane-Domain Mutations in Patients with Achondroplasia

	Independent Alleles		
	This Study	Previous Studies	Total (%)
1138 G to A	150	37	187 (96.9)
1138 G to C	3	2	5 (2.6)
No mutation	1	0	1 (.5)
Total	154	39	193 (100.0)

zon (Reardon et al. 1994) and Jackson-Weiss syndromes (Jabs et al. 1994), and a single FGFR1 mutation was discovered in five unrelated patients with Pfeiffer syndrome (Muenke et al. 1994). Craniosynostosis is the primary feature of each of these disorders, but Jackson-Weiss and Pfeiffer syndromes also have distal limb anomalies. It seems likely that other skeletal dysplasias will also be found to be caused by mutations in FGFRs.

FGFR3 is a prime candidate gene for at least two other skeletal dysplasias with features similar to achondroplasia. Hypochondroplasia (MIM 146000) is a condition with physical and radiographic findings (Hall and Spranger 1979) milder than those in achondroplasia. It is thought to be allelic with achondroplasia, on the basis of radiographic similarities and the existence of a hypochondroplasia/achondroplasia compound heterozygote with a severe phenotype (McKusick et al. 1973). There is also evidence that hypochondroplasia and achondroplasia may be linked to the same genetic location (LeMerrer et al. 1994; J. T. Hecht, unpublished data). Thanatophoric dysplasia (MIM 187600) is a lethal and most likely dominant form of dwarfism that has many radiographic features in common with achondroplasia (Gorlin et al. 1990). Chromosomal rearrangements involving 4p16 have been described in thanatophoric dysplasia and short rib–polydactyly syndrome (Urioste et al. 1994), which raises the possibility that FGFR3 may be involved in these syndromes. Preliminary data indicate that individuals with hypochondroplasia and thanatophoric dysplasia do not have either FGFR3 G380R mutation (data not shown).

The most remarkable finding of this study is the extremely high mutation frequency at the FGFR3 1138 guanosine nucleotide. Recurrent mutations have been reported for several other genes, including β globin and CFTR (Kazazian et al. 1984; Kulozik et al. 1986; Youssoufian et al. 1988; Green et al. 1990; Koeberl et al. 1990; Okano et al. 1990; Pattinson et al. 1990; Loux et al. 1991; Reiss et al. 1991; Grundy et al. 1992), but none approach the mutation frequency observed in achondroplasia. On the basis of a prevalence of achondroplasia estimated between 1/15,000 and 1/77,000 (Murdoch et al. 1970; Gardner 1977; Oberklaid et al. 1979; Andersen and Hauge 1989; Stoll et al. 1989), the mutation rate at the FGFR3 1138 guanosine nucleotide is between 5.5×10^{-6} and 2.8×10^{-5} per gamete per generation. The mutated nucleotide lies within a CpG dinucleotide. CpG dinucleotides have been associated with increased rates of mutation due to spontaneous deamination of a methylated cytosine (Cooper and Youssoufian 1988). Mutation rates for CpG-to-TpG transitions have been estimated to occur at a rate of 3.68×10^{-8} to 1.05×10^{-7} per gamete per generation (Green et al. 1990; Koeberl et al. 1990). The mutation rate of G-to-A transitions at FGFR3 1138 G is therefore between 50 and 760 times higher than previously estimated.

The reasons for the high rate of mutation at this particular nucleotide are not currently understood. It may be that this site is always methylated in the germ line and is exquisitely sensitive to deamination, thus leading to a high incidence of transition mutations. The G-to-A change results from conversion of cytosine to thymidine on the antisense strand, followed by incorporation of adenosine at the corresponding position, during replication of the coding strand (Cooper and Youssoufian 1988). Even if this particular CpG were always methylated, the rate of spontaneous deamination would have to be higher than that postulated elsewhere (Cooper et al., in press). Steinberg and Gorman (1992) have postulated the existence of an enzymatic deamination of methylated cytosine residues, to explain the high frequency of double mutations in the gene for the regulatory subunit of murine cyclic AMP-dependent protein kinase. An enzyme capable of catalyzing this reaction, *Hpa*II methylase, a DNA (cytosine-5) methyltransferase, has been isolated in prokaryotes and shown in vitro to cause a 10,000-fold increase in C-to-T transitions when S-adenosylmethionine was deficient (Shen et al. 1992).

The increase, over previous estimates, in the rate of transversions at this site is even more remarkable. The G-to-C mutation has been observed in five unrelated individuals and accounts for 2.6% of achondroplasia mutations characterized thus far. This implies a mutation rate of 6.96×10^{-7} to 1.37×10^{-6} per generation per gamete, 1,700–3,300 times higher than the estimated transversion rate of 4.1×10^{-10} per generation per gamete, determined by analysis of mutations in the factor IX gene (Koeberl et al. 1990).

The reasons for the high rate of transversion mutation at this nucleotide are unclear. Analysis of sequences surrounding the mutation reveals two CTT trinucleotide sequences located 8 and 11 bases downstream of nt 1138 (see fig. 1). CTT is the topoisomerase I cleavage-site consensus sequence (Bullock et al. 1985) and is found at an increased frequency in the vicinity of point mutations other than CpG transitions (Cooper et al., in press).

Although the rate of G-to-C transversion at position 1138 is extremely high, the G-to-T transversion has not been observed. Both the G-to-A and G-to-C mutations result in a glycine-to-arginine substitution in codon 380. A G-to-T transversion would result in a glycine-to-tryptophan substitution, which might present as a different phenotype or possibly be silent.

An alternative explanation for the high frequency of recurrent mutation in FGFR3 is a gene-conversion event. Gene conversions have been implicated in recurrent mutations in the genes for steroid 21-hydroxylase (Amor et al. 1988; Urabe et al. 1990: Collier et al. 1993), glucocerebrosidase (Sorge et al. 1990), C4 component of complement (Braun et al. 1990), and β-globin (Matsuno et al. 1992). Gene conversions require a donor sequence that is usually a closely linked pseudogene or other allele. The existence of an FGFR3 pseudogene has not been reported; however,

the structural organization of the FGFR3 gene has yet to be determined, and the question remains open as to whether a pseudogene exists.

Achondroplasia, like sickle-cell anemia and hemoglobin E disease, is one of the few genetic disorders known to be caused by a single, specific amino acid substitution. However, this observation is unprecedented for an autosomal dominant disorder in which new mutations account for >80% of cases. The FGFR3 1138 guanosine nucleotide is therefore the single most mutable nucleotide in the human genome reported to date. The explanation for this phenomenon remains to be determined.

Note added in proof.—Subsequent to the acceptance of the manuscript of this report, we have performed a first-trimester DNA-based prenatal diagnosis in a couple at risk for homozygous achondroplasia.

Acknowledgments

We wish to thank Mrs. Dee Miller, Mrs. Kay Smith, and the many people with achondroplasia who participated in these studies; Drs. Hal Dietz, Alan F. Scott, and Haig H. Kazazian Jr. for helpful discussions and review of the manuscript; and Dr. Jennifer Krupp for help with editing the manuscript. This work was supported by NIH grants R01AR41135, R01HD20691, and PO1HG00373, research grants 15,953 and 15,955 from the Shriners Hospital for Crippled Children, the Medical Genetics Branch of the National Center for Human Genome Research, and the Greenberg Center for Skeletal Dysplasias. G.A.B. is supported by an NIH training grant. R.I.O. is supported by Patrons of the Hospital Infantil de Mexico, Federico Gomez. Patients were seen in the General Clinical Research Centers of the Johns Hopkins University School of Medicine, including the Outpatient Clinical Research Center (MO1RR00722) and the Pediatric Clinical Research Center (MO1RR00052). Lymphoblast cell lines used in this study were established and maintained at the Kennedy Krieger Institute, with support from a NICHD Mental Retardation Research Center core grant (HD24061).

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